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CUPRESSUFLAVONE, A NEW BIFLAVONYL PIGMENT[1]

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Abstract—The leaves of *Cupressus torulosa* and *C. sempervirens* have been found to contain a new member of the biflavonyl group of natural colouring matters now named "cupressuflavone". On the basis of colour reactions, UV, IR, NMR spectral data and degradation, its structure has been deduced as 8,8"-biapigeninyl and confirmed by comparison of the compound and its derivatives with synthetic samples.

THE genus *Cupressus* (Cupressaceae) consists of twelve species distributed in North America, the Mediterranean regions and subtropical Asia at high altitudes. Five of them have been reported in India[2] viz. *C. torulosa*, *C. sempervirens*, *C. funebris*, *C. lusitanica* (*C. glauca*) and *C. macrocarpa*. *C. cashmeriana* has been considered to be only a form of *C. torulosa*. The Cupressaceae have been extensively investigated from taxonomical as well as commercial points of view, particularly because the heartwoods of these trees have good decay-resistant and insect-repellant properties. After the discovery that biflavonyl pigments are widely distributed in the leaves of the gymnosperms,[3-6] the leaves of a number of Cupressaceae were examined and hinokiflavone (I) was found in *C. funebris* and *C. arizonica*.[7] The object of the present investigation was a detailed study of the distribution of biflavonyls in Indian Cupressaceae (and also other conifers)[8,9] and the results obtained with the leaves of *C. torulosa* and *C. sempervirens* are reported now.

Cupressus torulosa (Himalayan Cypress) is indigenous to India. The tall trees yield the most durable coniferous wood in India; it requires no antiseptic treatment and is extensively used.[2] Ahluwalia and Seshadri[10] reported the presence of nootkatin in the heartwood and later studies revealed of a number of terpenes and tropolone

[1] Preliminary communication: V. V. S. Murti, P. V. Raman and T. R. Seshadri, *Tetrahedron Letters* No. 40, 2995 (1964).

[2] *Wealth of India* (*Raw Materials*) Vol. 2, p. 398. Council of Scientific and Industrial Research New Delhi, India (1950).

[3] W. Baker and W. D. Ollis, *Recent Developments in the Chemistry of Natural Phenolic Compounds* (Edited by W. D. Ollis) p. 152. Pergamon Press, Oxford (1961).

[4] N. Kawano, *Recent Progress in the Chemistry of Natural and Synthetic Colouring Matters and related fields* (Edited by T. S. Gore, B. S. Joshi, S. V. Sunthankar and B. D. Tilak) p. 177 Academic Press, New York (1962).

[5] W. Baker, Ref. 4 p. 187.

[6] W. Baker, A. C. M. Finch, W. D. Ollis and K. W. Robinson, *J. Chem. Soc.* 1477 (1963).

[7] T. Sawada, *J. Pharm. Soc. Japan* **78**, 1023 (1958).

[8] V. V. S. Murti and P. V. Raman, Proceedings of the Symposium on *Recent Advances in Plant Polyphenolics* National Institute of Sciences of India, New Delhi, (October 1964).

[9] V. V. S. Murti, in Proceedings of the Symposium on *Newer Trends in Taxonomy*, National Institute of Sciences of India, New Delhi, (January 1966).

[10] V. K. Ahluwalia and T. R. Seshadri, *Curr. Sci.* **23**, 154 (1954).

derivatives.[11-13] *C. sempervirens* (Mediterranean Cypress) is also found in the Himalayan region; its wood has insect-repellant properties and contains a number of terpenes. The leaves of *C. torulosa* and *C. sempervirens* used in the present investigation were collected from the forest area near Dehra Dun.

Extraction of the air-dried leaves of *C. torulosa* with boiling acetone yielded a new biflavonyl pigment named "cupressuflavone". It is yellow and does not melt below 360°. Its colour reactions and the UV spectrum suggest a flavonoid structure. The IR spectrum has bands at 3460 (OH) and 1655 cm^{-1} (chelated carbonyl). The presence of chelated 5-hydroxyl group(s) is also indicated by a green ferric reaction. The carbonyl band undergoes a bathochromic shift to 1640 cm^{-1} on methylation and to 1650 cm^{-1} on acetylation and this behaviour is characteristic of the chelated carbonyl group in flavonoids.[3,6,14,15]

Cupressuflavone and its derivatives retain water and solvents of crystallization tenaciously and hence satisfactory analysis is difficult. However elemental analysis, methoxyl value and the mol. wt. (ebullioscopic) of its methyl ether were in agreement with the formula $C_{30}H_{12}O_4(OMe)_6$ and this was supported by the analytical data of cupressuflavone, its acetate and the ethyl ether. From this it could be inferred that the pigment has a biflavonyl structure with six hydroxyl groups. The compound dissolves in aqueous sodium carbonate solution showing that acidic 7-hydroxyl group(s) are present. These inferences were further substantiated by bathochromic shifts of the short wavelength band in the UV spectrum in the presence of sodium acetate and aluminium chloride. It was considered likely that two 5-hydroxyl and two 7-hydroxyl groups are present in the biflavonyl. This was supported by the fact that cupressuflavone could be partially methylated to yield a tetramethyl ether showing that two hydroxyl groups were resistant to methylation; further, cupressuflavone hexamethyl ether underwent smooth partial demethylation to give the same tetramethyl ether. Degradation of the hexamethyl ether under a variety of conditions gave only anisic acid as the major identifiable product. The yield of the anisic acid made it probable that cupressuflavone possesses two 4'-hydroxyl groups in the two side-phenyl rings. Small amounts of other products were also formed but in spite of modification of the conditions of degradation their yields could not be improved. Attempts are being made to identify these and also degrade cupressuflavone and its derivatives by other methods.

From the above results cupressuflavone should be composed of two apigenin units joined by a C—C linkage. The biflavonyl compounds known earlier[3-6] belong to two types: the biphenyl ether pattern represented by hinokiflavone (I) and the biphenyl type represented by amentoflavone (II); both these are derived from apigenin. A comparison of the m.p. of cupressuflavone and its derivatives with those of the other two is given in Table 1. Amentoflavone referred to here has been obtained by the demethylation of its partial methyl ethers which are natural products.

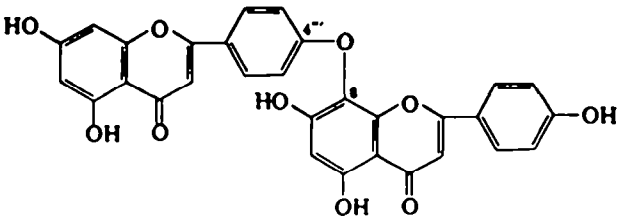
[11] W. Karrer, *Konstitution und Vorkommen der Organischen Pflanzenstoffe*, Birkhauser Verlag, Basel (1958).

[12] R. Heganauer, *Chemotaxonomie der Pflanzen* Vol. I. Birkhauser, Basel (1962).

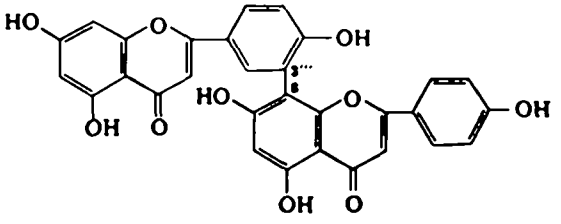
[13] H. Erdtman, *Chemical Plant Taxonomy* (Edited by T. Swain) p. 89 and earlier Refs. Academic Press, (1963).

[14] S. Balakrishna, J. D. Ramanathan, T. R. Seshadri and B. Venkatarmani, *Proc. Roy. Soc.* **268**, A, 1 (1962).

[15] J. D. Ramanathan and T. R. Seshadri, *Curr Sci.* 33, 553 (1964).



I



II

From the data it is clear that cupressuflavone is not identical with any of the earlier known biflavonyls and hence is probably a new type of biapigeninyl. Since no suitable degradation product which is useful in deciding the positions of linking between the two apigenin units could be isolated, the NMR spectrum of the cupressuflavone hexamethyl ether was examined (Fig. 1). This spectrum was taken at 60 Mc in deuterochloroform solution using TMS as internal reference. The assignments of the various proton signals are given in Table 2.

The NMR spectrum is clearly indicative of the symmetrical nature of linking between the two apigenin units. The positions of linking were inferred to be 8 and 8'' from the singlet at 6·60δ integrating to four protons. More recently it has been found that this signal resolves into two singlets when the spectrum is run at a slow speed (500 sec). No *meta* coupling is discernible in the spectrum which suggests that either the 6 or the 8 protons of the A-rings are absent. The signals of the 6- and the 3-protons in flavonoids and biflavonoids are usually found in this region while

TABLE 1. COMPARISON OF CUPRESSUFLAVONE WITH OTHER BIFLAVONYLS (M.P.S)

Derivative	Cupressuflavone	Amentoflavone[6,16]	Hinokiflavone[17]
Parent compound	360	>330	353–355
Methyl ether	295–297	227–228	259–260
Dioxime of the methyl ether	290–291	250	202–203
Ethyl ether	267–269	—	249–251
Acetate	252–254	242–244	239–240
Partial methyl ether (5,5''dihydroxy)	259–261	281–282	259–260
Diacetate of the partial methyl ether	168–170	230	255

[16] T. Kariyone and T. Sawada, *J. Pharm. Soc. Japan* **78**, 1016 (1958).
[17] T. Kariyone and Y. Fukui, *J. Pharm. Soc. Japan* **80**, 746 (1960).

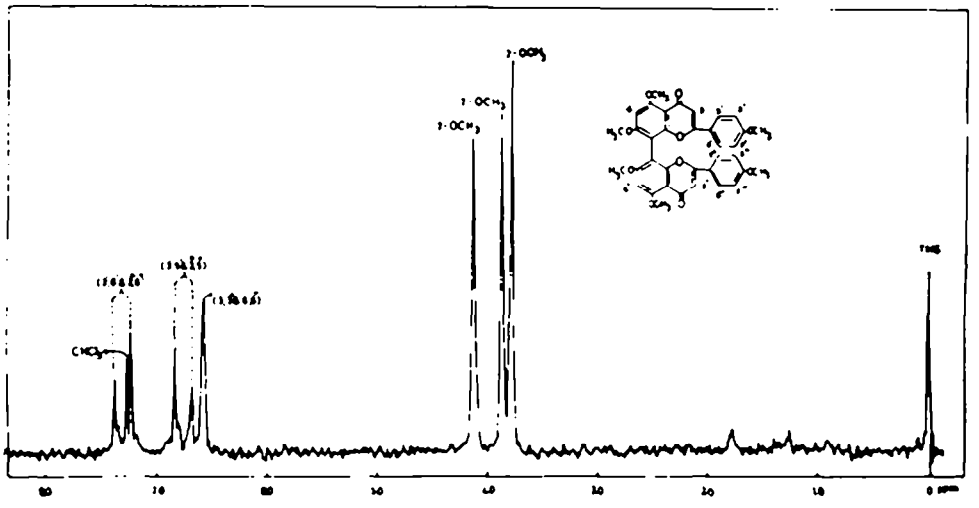


FIG. 1. NMR spectrum of the cupressuflavone hexamethyl ether.

those of the 8-protons generally appear at slightly lower fields.[18] Cupressuflavone itself should, therefore, be assigned the structure of 8,8"-biapigeninyl (IIIa).

8,8"-Biapigeninyl hexamethyl ether (IIIb) was earlier obtained by Nakazawa[19] as a byproduct in the synthesis of ginkgetin tetramethyl ether. A direct comparison of the synthetic sample with cupressuflavone hexamethyl ether by m.m.p., UV and IR spectra (Fig. 2) and paper chromatography showed that they are identical.

We have now repeated the synthesis of the hexamethyl ether from 8-iodoapigenin trimethyl ether using modified conditions for the Ullmann condensation. It was demethylated and the hexahydroxy compound thus obtained was acetylated. These have been found to be identical with cupressuflavone and its hexaacetate in all respects.

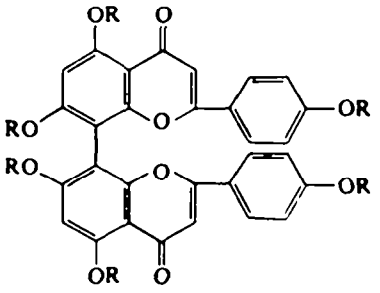
Two points of special interest about cupressuflavone need comment. The structure of this substance incorporates a diphenyl system in which all the *ortho* positions are substituted by oxygen atoms. However no optical activity could be detected either in the natural pigment or any of its derivatives. Other naturally occurring biphenyl type of biflavonyls (amentoflavone derivatives) have also been reported to be devoid

TABLE 2. CHEMICAL SHIFTS OF PROTONS IN CUPRESSUFLAVONE HEXAMETHYL ETHER

Signal (δ) ppm	Number of protons	J (cps)	Assignment
7·30 (d)	4	10	2', 6' and 2", 6" protons (*ortho* coupling)
6·70 (d)	4	10	3', 5' and 3", 5" protons (*ortho* coupling)
6·60 (s)	4	—	3,3" and 6" protons
4·15 (s)	6	—	two OMe groups
3·85 (s)	6	—	two OMe groups
3·75 (s)	6	—	two OMe groups

d = doublet; s = singlet

[18] T. J. Batterham and R. J. Highet, *Austr. J. Chem.* **17**, 428 (1964) and earlier references.
[19] K. Nakazawa, *Chem. Pharm. Bull.* **10**, 1032 (1962).



IIIa, R = H b, R = Me

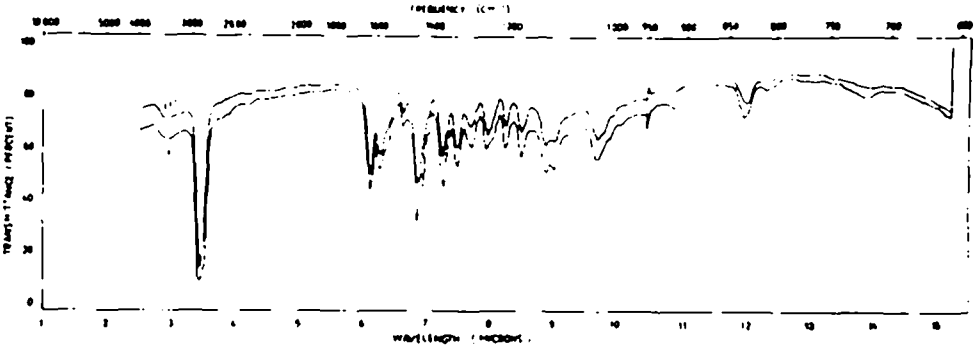
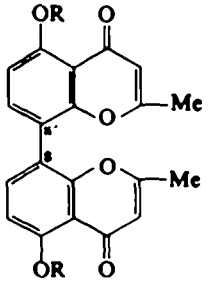
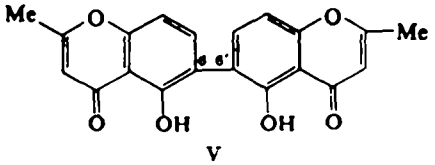


FIG. II. IR spectra of (1) cupressuflavone hexamethyl ether and (2) synthetic 8,8″-biapigeninyl hexamethyl ether.

of optical activity; in these compounds, however, all the ortho positions of the diphenyl residue are not occupied. The second point relates to the possibility of Wessely-Moser rearrangement in the biflavonyl group. In all these compounds one of the flavonoid units has the 5,7,8-substitution pattern and hence it could be expected that under appropriate conditions i.e. when refluxed with hydriodic acid for sufficiently long periods, rearrangement into the isomeric 5,6,7-pattern might occur. However our preliminary studies with cupressuflavone derivatives indicate that such an isomeric change is not easy to bring about. In this connection it might be mentioned that 2,2′-dimethyl-5,5′-dimethoxy-8,8′-bichromonyl (IVa) yields the normal demethylation product (IVb) with aluminium chloride in nitrobenzene but is isomerized to (V) when refluxed with hydriodic acid.[20] The results of our studies on these aspects will be reported later.





V

IVa, R = Me b, R = H

[20] B. Franck and G. Baumann, *Chem. Ber.* **96**, 3209 (1963).

EXPERIMENTAL

Isolation of cupressuflavone

Air-dried leaves (2 kg) of *C. torulosa* were extracted with hot acetone in a large soxhlet till the extract was almost colourless. From the combined extracts the solvent was distilled off and the remaining green semi-solid mass boiled with pet. ether (60–80°) (6 × 1 l. ×4 hr) in order to remove most of the chlorophyll and the waxes. The residue was refluxed with alcohol (6 times ×1 l.) when the crude cupressuflavone remained undissolved as a greenish-yellow solid Further purification was done by boiling with acetone and finally with water when the pigment was obtained as a yellow solid (15g). On crystallization from pyridine–MeOH it formed yellow small needles which did not melt below 360°. (Found: C, 64·7, 64·7; H, 4·1, 4·7; C-Me, nil; OMe, nil; $C_{30}H_{18}O_{10}$, H_2O requires: C, 64·7; H, 3·6%.)

Cupressuflavone is sparingly soluble in common organic solvents, moderately in chf and easily in pyridine and dimethylformamide. It gives a green ferric reaction and dissolves in Na_2CO_3aq, K_2CO_3aq and NaOHaq with a yellow colour. Its soln in conc H_2SO_4 is yellow without any fluorescence. On reduction with Mg and conc HCl in ethanolic soln it develops an orange colour. *UV absorption* (95% EtOH): 226 (4·68), 274 (4·58), 330 mμ (4·55); (Ethanolic AcONa): 231 (4·56) 285 (4·60), 335 mμ (4·33); (Ethanolic EtONa): 234 (4·61), 285 (4·60), 330 mμ (4·28); (Ethanolic $AlCl_3$): 231 (4·62), 281 (4·55), 304 (4·42), 341 mμ (4·51). *IR spectrum* (KBr, cm^{-1}): 3460, 1655, 1600, 1535, 1470, 1360, 1285, 1235, 1175, 1160, 1120, 1060, 910, 845, 830, 760, 735.

Cupressuflavone hexaacetate

Cupressuflavone (0·2 g) was heated with dry pyridine (3 ml) and Ac_2O (5 ml) at 100° for 3 hr and the soln was poured into crushed ice. The acetate (150 mg) crystallized from MeOH or AcOEt as colourless needles, m.p. 252–254°. (Found: 62·3; H, 4·8, CH_3CO, 30·7; $C_{42}H_{34}O_{16}$, H_2O requires: C, 62·4; H, 4·0; 6 CH_3CO, 31·9%.) $[\alpha]_D^{27}$, zero (*c*, 1·128, pyridine).

Cupressuflavone hexamethyl ether

Cupressuflavone (1 g) was dissolved in warm, purified dioxan (150 ml), dimethyl sulphate (redistilled, 5 ml) and freshly ignited K_2CO_3 (15 g) were added and the mixture was refluxed for 72 hr when the ferric reaction became negative. The crude product was dissolved in chf, the soln passed through a column of silica gel and the column washed with more chf. On removal of the solvent from the eluate the methyl ether was obtained as a yellow solid. It crystallized from MeOH or chf–acetone as pale yellow prisms, m.p. 295–297°; yield, 0·6 g. (Found: C, 69·5, 69·5; H, 5·2, 5·2; OMe, 26·0; C-Me, 0·2; M.W. (ebullioscopic in acetone), 585; $C_{36}H_{30}O_{10}$ requires: C, 69·5; H, 4·8; 6 OMe, 28·9%; M.W., 622.) UV (EtOH): 225 (4·75), 269 (4·68), 320 mμ (4·60). It gives an orange-red colour when reduced with Mg and conc HCl in alcoholic soln.

The identity of the cupressuflavone hexamethyl ether with synthetic 8,8″-biapigeninyl hexamethyl ether[19] was proved by m.m.p., comparison of the UV and the IR spectra and paper cochromatography (toluene: AcOH, 25:1 and 25:2; characteristic blue fluorescence in UV light). Further confirmation was provided by preparing the oxime of the cupressuflavone hexamethyl ether, by refluxing the latter in pyridine soln with hydroxylamine hydrochloride and AcOK for 3 hr; m.p. 290–291° (after crystallization from EtOH). Mixed m.p. of the oxime with cupressuflavone hexamethyl ether (m.p. 297°) was considerably depressed. Nakazawa reported[19] m.p. 294° for the oxime of the synthetic 8,8″-biapigeninyl hexamethyl ether.

Cupressuflavone-7,7″,4′,4″-tetramethyl ether

Method 1. A mixture of cupressuflavone (1 g), dimethyl sulphate (1 ml), K_2CO_3 (5 g) and dioxan or acetone (150 ml) was refluxed for 6 hr. The partial methyl ether formed pale yellow crystals from MeOH, m.p. 259–261°; yield, 0·6 g. (Found: C, 68·6; H, 4·7; OMe, 18·8; $C_{34}H_{26}O_{10}$, $\frac{1}{2}H_2O$ requires: C, 68·7; H, 4·3; 4 OMe, 19·9%.) It gives a green ferric reaction.

The diacetate of the tetramethyl ether, prepared by the Ac_2O–pyridine method, crystallized from AcOEt as colourless crystals, m.p. 168–170°.

Method 2. A soln of the cupressuflavone hexamethyl ether (0·2 g) in dry nitrobenzene (20 ml) was treated with anhyd $AlCl_3$ (95 mg) and the mixture heated on a boiling water-bath for 1½ hr. After cooling HCl (1:1, 50 ml) was added and the nitrobenzene removed by steam-distillation.

The product (0·1 g) was crystallized from MeOH; m.p. 259–261°. Mixed m.p. with the compound prepared by the Method 1 was not depressed.

Cupressuflavone hexaethyl ether

Cupressuflavone (0·5 g) was suspended in dry acetone (100 ml), diethyl sulphate (redistilled, 2 ml) and ignited K_2CO_3 (8 g) were added and the mixture refluxed for 72 hr. The ethyl ether was purified as in the case of the hexamethyl ether and then crystallized from MeOH; m.p. 267–269°, yield, 0·3 g. (Found: C, 70·1; H, 6·0; $C_{48}H_{42}O_{10}$, H_2O requires: C, 69·6; H, 6·1%.)

Degradation experiments with cupressuflavone hexamethyl ether

(i) *with alkaline hydrogen peroxide.* To a stirred mixture of the methyl ether (0·5 g) and 40% KOHaq (40 ml), 30% H_2O_2 (10 ml) was added slowly during 1 hr and stirring was continued for another 3 hr at room temp. After allowing to stand for 42 hr another 10 ml H_2O_2 soln was added and a further 8 ml after 50 hr, the mixture being stirred occasionally. After a total period of 72 hr the unchanged methyl ether was filtered, the filtrate was acidified with dil H_2SO_4 and continuously extracted with ether. The ether soln was evaporated, the residue dissolved in chf and the soln successively extracted with satd $NaHCO_3$aq (4 × 25 ml), 5% Na_2CO_3aq (5 × 10 ml) and 2% NaOHaq (5 × 10 ml). The extracts were filtered, acidified, continuously extracted with ether and the solvent removed from the ether extracts. Only small amounts of the degradation products were obtained from the Na_2CO_3 and the NaOH fractions. The solid obtained from the $NaHCO_3$ soluble fraction was purified by redissolution in $NaHCO_3$aq and precipitation by acidification. After recrystallization from hot water (charcoal) it melted at 178–180° alone or on admixture with an authentic sample of anisic acid, yield, 100 mg.

(ii) *with absolute ethanolic potash.* Cupressuflavone hexamethyl ether (1 g) was refluxed with abs ethanolic KOH (12%; 50 ml) for 6 hr, the alcohol was distilled off and water (200 ml) added to the residue. The yellow solid (0·2 g) was filtered, washed with water and crystallized from chf–MeOH mixture; m.p. 294–296°. This was identified as the unchanged original methyl ether.

The alkaline filtrate was acidified and worked up as in the previous degradation. The Na_2CO_3 and the NaOH fractions were small and from the $NaHCO_3$ fraction anisic acid was isolated, m.p. and m.m.p. 180–181°.

(iii) *with methanolic baryta.* The hexamethyl ether (1·5 g) was refluxed with methanolic baryta soln (8%; 100 ml) for 6 hr, the solvent was removed and water (100 ml) added. The unchanged methyl ether that separated was filtered; m.p. 293–295°, 0·5 g. The alkaline filtrate was worked up as in the previous experiments and from the $NaHCO_3$ soluble fraction anisic acid (m.p. 180–182°) was isolated. The Na_2CO_3 and the NaOH soluble fractions were small and could not be fully identified.

(iv) *with neutral permanganate.* The methyl ether (4·2 g) was dissolved in purified, hot acetone (250 ml), $MgSO_4.7H_2O$ (2 g) was added and the mixture was gently refluxed on a water-bath; a soln of $KMnO_4$ (1·8 g in 100 ml purified acetone) was slowly added during 2 hr. After refluxing for another 2 hr the solvent was distilled off and the residue treated with $NaHSO_3$ and dil H_2SO_4 to decompose the MnO_2 and the excess permanganate. After keeping in the refrigerator overnight the mixture containing some solid was extracted with chf and the soln fractionated with $NaHCO_3$, Na_2CO_3 and NaOH solns. The compound insoluble in all the 3 solutions was recovered by removing chf and crystallized from chf–MeOH; m.p. 292–294°, 3·5 g. This was found to be the unchanged original methyl ether. The bicarbonate soluble fraction was identified as anisic acid and only small quantities of the material could be obtained from the carbonate and the hydroxide soluble fractions.

Ullmann condensation of 8-iodo-apigenin trimethyl ether: synthesis of cupressuflavone hexamethyl ether

A mixture of 8-iodo-apigenin trimethyl ether[19] (2·2 g) and activated Cu bronze (1·8 g) was heated at 245–255° for 1½ hr. The solid cake was cooled, powdered, exhaustively extracted with chf in a soxhlet and the solvent removed to yield a red-brown residue. This gave a greenish-brown ferric reaction showing that demethylation had taken place during the condensation.* The crude

* Similar observation was also made by Professor Nakazawa (private communication to Professor Seshadri).

reaction product was methylated by shaking with dimethyl sulphate (8 ml) in ethanolic soln (10 ml) with gradual addition of 40% KOHaq till the mixture was alkaline. After 1 hr the greenish-yellow solid was filtered off, washed free from alkali, then with dil AcOH and finally with water. The residue was dried, dissolved in hot benzene, the soln filtered through a column of silica gel and the column eluted with more benzene. The yellow soln on removal of the solvent left a yellow solid which crystallized from chf-MeOH as yellow prisms, m.p. 293–295°; 0·4 g. This was identical with the cupressuflavone hexamethyl ether (m.m.p. and paper cochromatography).

8,8″-Biapigeninyl

A soln of the above 8,8″-biapigeninyl hexamethyl ether (0·2 g) in dry benzene (50 ml) was treated with anhyd $AlCl_3$ (1 g) and the mixture was refluxed on a steam-bath for 2 hr. The solvent was removed and the yellow residue treated with ice and strong HCl (10 ml) and heated for a few min. The solid, after crystallization from pyridine–MeOH, decomposed at 355–360° and gave a green ferric reaction, yield, 0·1 g. The acetate (Ac_2O–pyridine) formed colourless crystals from AcOEt, m.p. 250–252°. 8,8″-Biapigeninyl and its acetate were identical with cupressuflavone and its hexaacetate in all respects.

Isolation of cuperssuflavone from C. sempervirens

Air-dried leaves of *C. sempervirens* (2·5 kg) were extracted with hot acetone and the acetone concentrate worked up as described in the case of the *C. torulosa* extracts. Cupressuflavone was obtained as a yellow solid (5 g) and did not melt below 360° (pyridine–MeOH). It was identical with the compound isolated from the leaves of *C. torulosa* in colour reactions, UV and IR spectra and gave the same hexamethyl ether and hexaacetate.

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